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RESEARCH ARTICLE

Evaluation of genetic diversity in different populations of six *Salvia* species using R-ISSR markers analysis

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Abstract Genetic diversity assessment is pivotal to comprehend the evolutionary dynamics, and conservation implications of plant species. This study used R-ISSR markers to evaluate the genetic diversity of six *Salvia* species (*S. verticillata*, *S. glutinosa*, *S. reuteriana, S. hypoleuca, S. leriifolia, and S. limbata*) across multiple populations in Iran. We sampled 28 populations (92 specimens), and conducted DNA analysis using selected R-ISSR markers to assess genetic variation. After screening 10, R-ISSR primers, four primers that produced clear and reproducible fragments were selected for further analysis. The four primers generated 93 bands ranging in the size from 100 to 2000 bp, averaging 23.25 bands per primer. Polymorphic information content (PIC) values ranged from 0.223 to 0.260, and marker index (MI) values ranged from 4.43 to 6.02 per primer. Moreover, P9 exhibited the highest PIC (0.260), and P1 displayed the highest MI (6.02). The mean coefficient of gene differentiation (Gst) was 0.7014, signifying that 48% of genetic diversity was within populations. This research yields insights into genetic differentiation, population structure, limited gene flow, marker utility, and genetic affinities. Significant differentiation between and within *Salvia* populations is indicated by the observed polymorphism and genetic diversity, which are most likely the result of elements like geographic isolation, environmental variations, and reproductive obstacles. Clustering analysis shows distinct genetic clusters, and subpopulations within each *Salvia* species. Moreover, the results from the analysis of molecular variance highlight significant genetic variation among populations, thereby suggesting limited gene flow, which was potentially affected by geographical barriers and ecological factors. Particularly, highly polymorphic P1 and P9 primers offer valuable tools for future breeding research, and germplasm management in *Salvia* species. Furthermore, our findings show genetic affinities, and relatedness among specific populations, thus indicating closer relationships between certain *Salvia* species.

Keywords Cluster analysis · Endemic · Genetic diversity · R- ISSR Markers · *Salvia* species

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Introduction

Salvia, boasting more than 1890 species from old and new world, holds the distinction of being the largest genus within Lamiaceae family (IPNI).

Iran stands as a significant repository of *Salvia* L. germplasm, with approximately 61 species of annual and perennial herbaceous plants belonging to *Salvia* genus. Furthermore, 17 of these species are endemic to the country (Mozaffarian 2004). The *Salvia* genus

has 1893 known species worldwide (IPNI), and the plants may be found thriving in a variety of climates, from temperate to semitropical to arctic, including tropical areas in Iran. Consequently, inter-specific hybridization contributes to a remarkable morphological diversity (Hedge 1982).

Salvia, commonly known as sage or Chia, has garnered attention in recent research in terms of its numerous medicinal properties (Valizadeh et al. 2014a, b; Mahmoudi Kordi et al. 2015). The genus was associated with various biological activities, such as antioxidant, antifungal, cytotoxic, antibacterial, antitumor, antiprotozoal, and HIV inhibitory effects (Ulubelen 2003; Imanshahidi and Hosseinzadeh 2006). Therefore, certain *Salvia* species hold economic importance as the sources of essential oils and aroma chemicals for the perfume industry (Kamatou et al. 2008). These species produce active natural compounds falling into categories, such as terpenoids, steroids, flavonoids, and polyphenols (Shirota et al. 2006).

The survival and adaptability of plant species depend critically on genetic variation because it enables them to adjust to changing environmental conditions and retain their long-term viability. Understanding the genetic diversity within plant populations is crucial for conservation efforts, and the development of effective management strategies.

Three significant factors contribute to genetic diversity in sage: selection, geographical distribution, and speciation. Alongside open and cross-pollination, these factors create uncertainty in the taxonomy and genetic relationships within *Salvia* genus (Wang et al. 2011). Species identification based solely on morphological characteristics is challenging, as environmental conditions easily influence such traits.

The basic chromosome numbers of *Salvia* species were classified into five groups: x = 7, 8, 9, 10, and 11. The ploidy levels vary among different species, ranging from diploid to tetraploid (Hesamzadeh Hejazi 2016; Kursat et al. 2018). The chromosome counts for the species studied in this research are as follows: *S. verticillata* (2n = 4x = 32), *S. glutinosa* (2n = 2x = 16), *S. reuteriana* (2n = 2x = 20), *S. hypoleuca* (2n = 2x = 22), *S. leriifolia* (2n = 2x = 22), and *S. limbata* (2n = 2x = 22) (Hesamzadeh Hejazi and Ziaei Nasab 2023).

Regarding the importance of preserving and using plant genetic resources, understanding the genetic characteristics, and the diversity of plants, particularly in highly diverse genera like *Salvia*, are essential. In the era of modern biotechnology, DNA-based molecular markers offer cost-effective, stable, abundant, highly polymorphic, and environmentally-independent tools for identifying cultivars and evaluating genetic relationships among plant species.

Various molecular markers were used in *Salvia*-related research, including random amplified polymorphic DNA (RAPD), inter simple sequence repeats (ISSR), molecular phylogenetics, nrDNA ITS sequences, DAMD-PCR, PCR–RFLP, and simple sequence repeats (SSR) markers (Erbano et al. 2015; Liber et al. 2014; Zhang et al. 2013a, b; Song et al. 2010; Javan et al. 2012; Zhang et al. 2012; Karaca et al. 2008; Radosavljević et al. 2012; Walker et al. 2007).

We used R-ISSR markers, which involve the amplification of DNA regions via multiplex PCR reactions using a suitable combination of RAPD and ISSR primers. The number of amplified R-ISSR products, including novel genomic loci, depended on the combination of RAPD primers employed in the research (Smolik 2012).

During PCR genomic amplifications, using ISSR and RAPD primers in the combination yielded three types of amplified products: RAPD amplicons (obtained by amplifying DNA with a single RAPD primer as forward and reverse primers), ISSR amplicons (obtained by amplifying DNA with a single ISSR primer as forward and reverse primers), and hetero-amplicons (obtained by amplifying DNA with ISSR and RAPD primers simultaneously).

R-ISSR markers have emerged as a significant tool in genetic diversity research owing to their capacity to create high-resolution DNA profiles by amplifying sections of the genome that contain simple sequence repeats. This technique allows for the identification of polymorphic loci within the genome, enabling researchers to assess genetic variability at the population level. Using R-ISSR markers,

the objectives of our research were to investigate the genetic diversity within, and among different populations of two endemic species and four native species in Iran, as well as to demonstrate the potential application of R-ISSR technique as a screening tool to describe genetic variations. We examined a total of 28 populations (92 specimens) from six different *Salvia* species to establish breeding programs. Considering

the potential threats to Iran's natural areas, the sage plant is one of the endangered species requiring urgent evaluation of its diversity, particularly in populations that hold economic value.

Materials and methods

Plant Materials and DNA Extraction

To assess the genetic diversity of six *Salvia* species (*S. verticillata, S. glutinosa, S. reuteriana, S. hypoleuca, S. leriifolia, and S. limbata)* in Iran, we collected plant samples from multiple populations across different geographical regions. A total of 28 populations were included, with 92 individuals sampled from all populations. Care was taken to ensure representative sampling across the species distribution range in Iran (Table 1).

Genomic DNA was isolated from the collected fresh young leaves samples using a modified cetyl trimethyl ammonium bromide (CTAB) protocol (Doyle and Doyle 1987). Approximately 0.6 g of leaves were ground into a fine powder in a mortar using liquid nitrogen. The powdered tissue was transferred to a 2 ml Eppendorf tube containing 900 µl of the extraction buffer (100 mM Tris–HCl, 50 mM EDTA, 1.5 M NaCl, 0.2% mercaptoethanol, 2.5% (w/v) CTAB, 0.35 M sorbitol, 1% Triton X-100, and 1.5% PVP). The homogenate was then incubated at 65 °C for 90 min with mixing every 5 min. The homogenate was subsequently extracted with 800 µl of chloroform/isoamyl alcohol (24:1), and centrifuged at 8,000 rpm for 10 min. The supernatant was carefully transferred to a labeled new tube, and 200 µl of NaCl (5 M) was added. Following a 10-min centrifugation at 8,000 rpm, the supernatant was transferred to a fresh tube and 1/4 (v/v) LiCl was added. After a few hours of vigorous mixing by inversion, the mixture was centrifuged for 20 min at 10,000 rpm. The supernatant was transferred to a new tube, and 2/3 (v/v) cold isopropanol (70%) was added. After mixing for a few minutes, the mixture was centrifuged at 11,000 rpm for 11 min. The supernatant was discarded, and the pellet was washed with 400 µl of 70% ethanol. Following centrifugation at 14,000 rpm for 3 min, the supernatant was discarded, and the pellet was dried in an Eppendorf Vacfuge™ at 37 °C for 15 min. The pellet was then re-suspended in 200 µl of TE (10:1) buffer and 5 µl of RNase A solution (100 mg/ml) was added. The mixture was thoroughly mixed, and incubated at 37 °C for 1 h. The homogenate was again extracted with an equal volume of chloroform/isoamyl alcohol (24:1), followed by centrifugation at 12,000 rpm for 10 min. The supernatant was transferred to a new 1.5 ml clear-colored tube, and 2/3 (v/v) cold isopropanol (70%) was added. After mixing for a few minutes, the mixture was centrifuged at 14,000 rpm for 10 min. The supernatant was discarded, and 400 µl of 70% ethanol was added to each tube to wash the pellet. After the centrifugation at 14,000 rpm for 3 min, the supernatant was discarded, and the pellet was dried in an Eppendorf Vacfuge™ at 37 °C. The dried pellet was then re-suspended in 200 µl of TE (10:0.1) buffer and stored at -80 °C until further use.

DNA quantity and quality

Purified DNA samples were quantified using a BioQuestTM CE2502 Spectrophotometer (CECIL Instrument Limited, Milton Technical Center, Cambridge, England). DNA concentration was measured by determining the absorbance ratios (A260/A280), A230, and A320. A pure DNA sample typically has an A260/A280 ratio of 1.8, indicating minimal protein contamination. The purity of the isolated total DNA was subsequently evaluated by 0.8% agarose gel electrophoresis. For further analysis, the DNA samples were diluted to a 5 ng/µl concentration and kept at −20 °C.

Primers and assays

For the genetic analysis, we used R-ISSR molecular marker technique. R-ISSR markers were selected based on their ability to produce clear and reproducible banding patterns.

Ten pairs of primers (Table 2), purchased in lyophilized form from TIB MOLBIOL-Germany, were tested for their ability to amplify DNA. Among them, four pairs of primers that showed significant polymorphism and reproducibility were selected for further analysis.

Polymerase chain reaction (PCR) amplifications were performed in a 50 µl reaction volume containing 1X PCR buffer, 1 mM MgCl2, 0.2 mM each of dNTPs, 0.7 µM of each primer, 2.5 U of Taq DNA

Table 1 Geographical characteristics of a sampled population of six species of *Salvia* genus

Population	Species	Locality	Altitude (m)	Latitude / longitude
Pop1	*S.glutinosa(k1)*	Klardasht1	1126	N: 36,34,9.4 E: 51,09,51.2
		Klardasht1	1134	N: 36,34,13.2 E: 51,09,52.6
		Klardasht1	1132	N: 36,34,12.3 E: 51,09,53.4
Pop2	*S.glutinosa(k2)*	Klardasht2	1045	N: 36,34,42.5 E: 51,09,23.6
		Klardasht2	1044	N: 36,34,45.4 E: 51,09,25.4
		Klardasht2	1042	N: 36,34,43.4 E: 51,09,24.5
Pop3	*S.glutinosa(S2)*	Guilan1	338	N: 37,02,24.3 E: 49,53,49.3
		Guilan1	333	N: 37,02,26.6 E: 49,53,49.5
Pop4	*S.glutinosa(S1)*	Guilan2	1475	N: 36,56,31.3 E: 49,53,38.8
		Guilan2	1473	N: 36,56,37.8 E: 49,53,38.9
		Guilan2	1472	N: 36,56,26.5 E: 49,53,39.6
Pop5	*S. verticillata(ch)*	Tehran-Lavasan	1819	N: 35, 52, 13.7 E: 51, 32, 24.3
Pop6	*S. verticillata(sh)*	Chalus- Sira village	1839	N: 36, 01, 22.1 E: 51,09, 6.7
Pop7	*S. verticillata(va)*	Firouzkouh-Tangehvashei	2219	N: 35, 51, 39.3 E: 52, 43, 37.5
Pop8	*S.reuteriana(ba)*	Karaj-Baraghan	1661	N: 35,53,55 E: 50,58,32.4
Pop9	*S.reuteriana(vard)*	Karaj-Vardavard	2243	N: 35,47,18.7 E: 51,06,52.6
Pop10	*S.reuteriana(phy)*	Firoozkooh	2169	N: 35,39,18 E: 52,12,42.2
Pop11	*S.reuteriana(sh)*	Tehran-Shemshak	1819	N: 35,52,13.7 E: 51,32,24.36
Pop12	*S.reuteriana(AZ)*	Azerbaijan-Shahindej	1500	N: 46,31,44 E: 36,56,12
Pop13	*S. hypoleuca(1)*	Karaj-Vardavard	2178	N: 35,44,16 E: 51,07,51
Pop14	*S. hypoleuca(2)*	Karaj- Aghesht	1510	N: 36,0,5.3 E: 50,52,15
Pop15	*S. hypoleuca(3)*	Karaj- Chalus road	1470	N:36,39,15 E:51,25,16
Pop16	*S. hypoleuca(4)*	Vardavard- Dakal road	1224	N:35,25,66 E:51,12,56
Pop17	*S. hypoleuca(5)*	Firouzkouh-Tangehvashei	2200	N:35, 48, 35 E:52, 36, 24.5
Pop18	*S. hypoleuca(6)*	Qazvin(1)	1830	N:36,24,22 E:51,13,35
Pop19	*S. hypoleuca(7)*	Qazvin(2)	2168	N:36,22,46 E:50,12,40

Table 1 (continued)

Population	Species	Locality	Altitude (m)	Latitude / longitude
Pop20	*S. hypoleuca(8)*	Karaj	2127	N:36,13,05 E:50,46,06
Pop21	*S. hypoleuca(9)*	Semnan	1236	N:35,36,12 E:53,00,01
Pop22	*S. leriifolia(1)*	Sabzevar(1)	1080	N:36,12,46 E:56,32,40
Pop23	*S. leriifolia(2)*	Bajestan(1)	1350	N:34,28,70 E:58,72,58
Pop24	*S. leriifolia(3)*	Sabzevar(2)	1000	N:36,00,38 E:57,42,17
Population	Species	Locality	Altitude (m)	Latitude / longitude
Pop25	*S. leriifolia(4)*	Bajestan(2)	1150	N:36,29,34 E:58,07,30
Pop26	*S. limbata(1)*	Meymeh	1942	N:33,29,34 E:51,11,30
Pop27	*S. limbata(2)*	Karaj	1467	N:35,49,43 E:50,58,9.8
Pop28	*S. limbata(3)*	Karaj-Aghesht	1550	N:36,0,5.3 E:50,52,15

Table 2 R-ISSR primers with their sequences and annealing temperature (TM), Y = C/T

Code No	Primer	Sequence(5'-3')	Annealing Temp.(◦C)
P1	UBC834 & U23	$(AG)_8$YT CCCGCCTTCC	41.0
P4	UBC808& U23	$(AG)_8$C CCCGCCTTCC	40.0
P8	UBC823& U23	$(TC)_8$C CCCGCCTTCC	42.0
P9	UBC820& D01	$(GT)_8$C ACCGCGAAGG	43.0
P12	UBC823& U04	$(TC)_8$C CCTGGGCTGG	43.0
P15	UBC875& D01	$(CTAG)_4$ ACCGCGAAGG	42.0
P20	UBC864& D08	(ATG)6 GTGTGCCCCA	43.0
P16	UBC876& A17	$(GATA)_2(GACA)_2$ GACCGCTTGT	42.0
P25	UBC815& D01	$(CT)_8$G ACCGCGAAGG	42.0
P26	UBC811& D03	$(GA)_8$C GTCGCCGTCA	42.0

polymerase (Qiagen-Germany), 5% formamide, and 12 ng of template DNA.

PCR amplification was carried out using an Eppendorf Mastercycler gradient Thermal Cycler under the following cycling conditions: an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at specific temperatures depending on the primers (refer to Table 2) for 1 min, and extension at 72 °C for 1.5 min. A final extension step was performed at 72 °C for 10 min. The resulting amplicons were separated using agarose gels electrophoresis (1%) run at 50 V in 1×TBE buffer, visualized by ethidium bromide staining, and photographed under ultraviolet light. The molecular weights of amplified fragments were estimated using a 100 bp DNA ladder.

Data analysis

The obtained DNA profiles were visualized, documented, and scored for the presence or absence of bands. The data were then used to construct a binary matrix, representing the genetic variation among individuals and populations.

In order to evaluate each primer's ability to identify polymorphic loci in both populations and individuals, Marker Indices (MI) for the R-ISSR markers were calculated using the technique outlined by Milbourne et al. (1997). The polymorphic information content (PIC) value was calculated using the formula

$PIC_i = 1 - \sum Pi^2$ where Pi is the frequency of the bands, as proposed by Roldan-Ruiz et al. (2000). The software programs POPGENE 1.31 (Yeh et al. 1997) and GenAlex (Peakall and Smouse 2006) were used to calculate single population gene frequencies and grouped population gene frequencies, as well as Nei's (1972) genetic distances matrix based on the binary data matrix. The distance matrix obtained was used to construct a Weighted Neighbor-Joining (WNJ) phenogram for the 28 populations using DARwin V5.0.158 software (http://darwin.cirad.fr/).

Additional genetic diversity parameters, including the observed number of alleles (na), number of effective alleles (ne), Nei's (1973) genetic diversity index (h), Shannon's Information index (I), total heterozygosity (Ht), expected heterozygosity within subpopulations (Hs), the coefficient of genetic differentiation (Gst), and the estimate of gene flow from Gst (Nm) were analyzed using POP GENE, and GenAlex software. R-ISSR data were subjected to a hierarchical analysis of molecular variance (AMOVA) following the method described by Excoffier et al. (1992). The AMOVA analysis was performed using GenAlex software.

Additionally, GenAlex software was used to perform a Principal Coordinates Analysis (PCOA) to illustrate the connection between the components of the distance matrix based on their first two principal coordinates.

Results

Using R-ISSR as a cost-effective and efficient method for genotype identification, and genetic relationship assessment within and among populations was found to be promising. Among 10, R-ISSR primers surveyed across *Salvia* species, only four primers showed considerable polymorphism and reproducibility which were selected for further analysis (Table 2 and Fig. 1).

A total of 28 populations from six different *salvia* species were analyzed using R-ISSR primers, resulting in the amplification of 93 scorable bands ranging in size from 100 to 2000 bp. On average, each primer yielded 23.25 bands (Table 3 and Fig. 1). The population with the highest number of scored bands was *S. hypoleuca*(Pop15), of which 41.94% were polymorphic. This was followed by *S. glutinosa* (Pop4) with 37.63% polymorphic bands. The population with the least polymorphism (5.38%) was observed in *S. limbata* (Pop26).

The range of polymorphic information content (PIC) values, which serve as indicators of the markers' ability to discriminate, was found to be between 0.223 and 0.260. Similarly, the marker index (MI) varied from 4.43 to 6.02 per primer. Among the primers, P9 showed the highest PIC value (0.260), and P1 had the highest MI value (6.02) (Table 3). Primer P9 $(GT)_8C + ACCGCGAAGG$ stood out as the best primer in terms of the sharpness of its patterns, and the high number of polymorphic markers it provided.

The average values of gene diversity (h) and Shannon's Information index (I) for 92 individuals across different populations, determined based on four R-ISSR markers, were found to be 0.236 ± 0.144 and 0.381 ± 0.187, respectively (Table 4). The highest values for (h) and (I) among the 92 individuals were observed for the P1 marker (0.4996 and 0.6927, respectively). The mean values of effective alleles, gene diversity, Shannon's index, total Heterozygosity (Ht), Expected Heterozygosity within subpopulations (Hs), coefficient of genetic differentiation (Gst), an estimate of gene flow from Gst (Nm), based on R-ISSR data, were 1.36, 0.24, 0.38, 0.25, 0.074, 0.701, and 0.213, respectively (Table 4).

Among the loci, P1-G showed the highest number of effective alleles (1.99), Shannon's index (0.693), and gene diversity value (0.499), while P4-E had the highest Total Heterozygosity (0.496) and P8-H had the highest Expected Heterozygosity (0.221) (Table 4). The gene flow, estimated as Nm = 0.5(1—Gst)/Gst, among the 93 scorable bands, ranged from 0.02 to 3.07 (Table 4).

The genetic identity values determined using Nei's (1987) unbiased measures varied from 0.624 between *S. hypoleuca* (Pop19-20) and *S. limbata* (Pop27) to 0.995 between *S. reuteriana* (Pop8—Pop10) and *S. reuteriana* (Pop9—Pop11).

The mean coefficient of gene differentiation (Gst) was 0.701 and the value of (φ_{ST}) was 0.522, indicating that 48% of the genetic diversity resided within the populations (Table 5). In addition, the examination of population genetic structure revealed the presence of discrete genetic clusters and subpopulations within every Salvia species. The 28 Salvia populations were categorized into six primary groupings using clustering analysis, which used unweighted

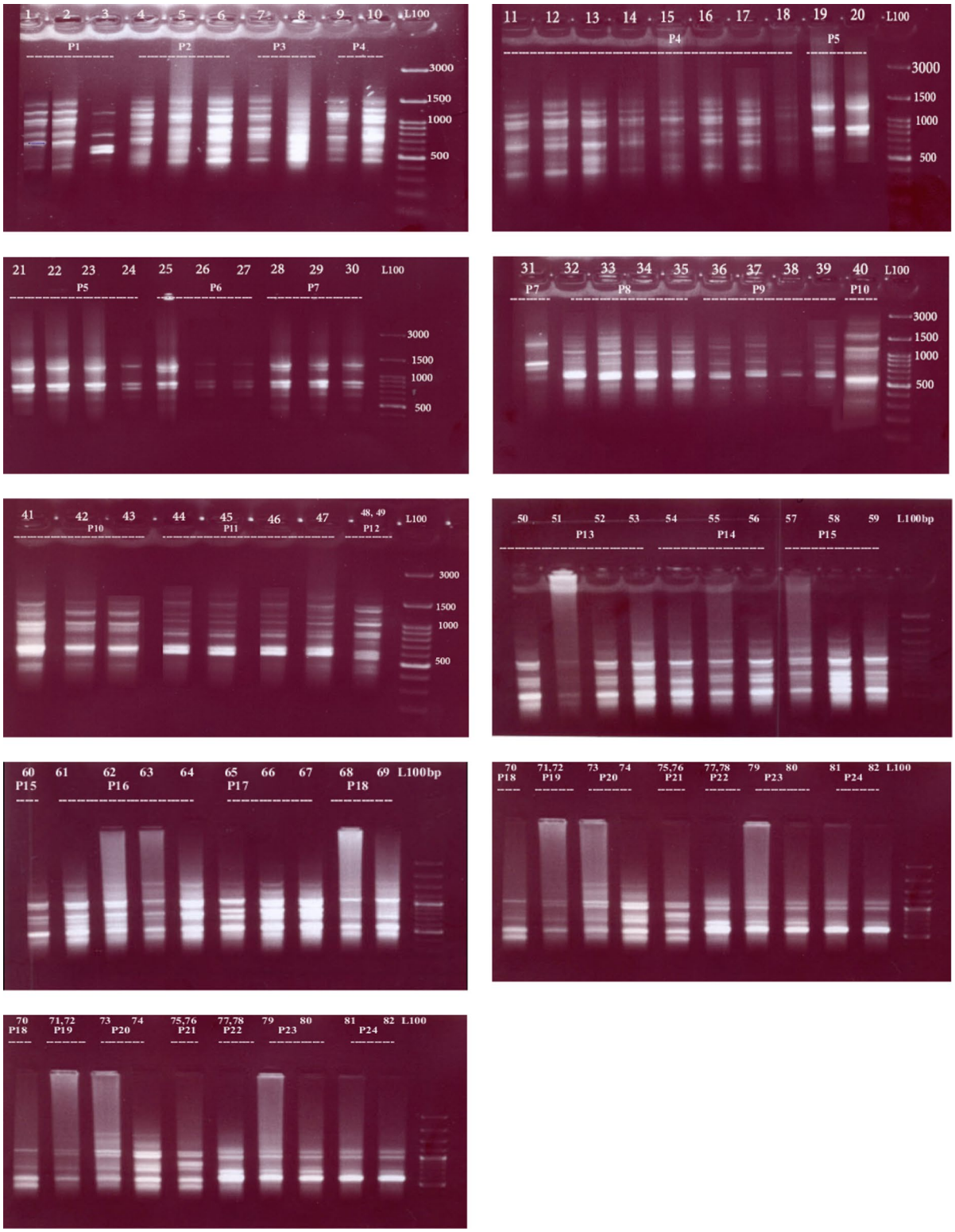

Fig. 1 R- ISSR marker profiles of 28 populations (92 individuals) of *Salvia* species (according to Table 1) generated by just one of the best primer (P9) in 1% agarose gel. Lane (L): 100 bp DNA ladder

Table 3 The number of total and polymorphic bands amplified in 28 populations with polymorphism information content (PIC) and marker index (MI) values using the best R-ISSR primers in *Salvia* species

MI	PIC	polymorphism %	Number of polymorphic bands	Band amplitude produced	Total number ofbands	Sequence (5' -3')	Primer
6.02	0.223	100%	27	200–2000	27	$(AG)_8$YT + CCCGCCTTCC	P1
4.43	0.233	100%	19	100–1500	19	$(AG)_8$C + CCCGCCTTCC	P4
5.54	0.231	100%	24	400–1800	24	$(TC)_8$C + CCCGCCTTCC	P8
5.98	0.260	100%	23	400–1700	23	(GT)8C + ACCGCGAAGG	P9
5.49	0.237		93 23.25		93 23.25		Total Mean

neighbor-joining based on Nei's unbiased measurements of genetic distance. The cophenetic correlation of single tree was approximately $r = 0.81$, indicating a relatively good fit among the genetic distances calculated and the dendrogram. The analysis showed a high similarity among *S.verticillata* populations, indicating their close relatedness. The sub-clustering pattern of the cluster correctly grouped all populations except for one population, *S. reuteriana*(AZ), which showed a mismatch with the initial identification of the population. This mismatch may be attributed to the inclusion of genotypes bred for specific objectives and collected from different geographical locations may have contributed to the clustering of most cultivars or populations in a single cluster. Hence, Hedge (1982) has divided *Salvia* species into five groups, and two species *S. glutinosa* and *S. reuteriana* are in the same group—the isolation of certain species such as *S. glutinosa* (S2), and *S. verticillata* (va). *S. hypoleuca*(5) *and S. hypolecuca*(8) are likely attributed to the presence of a specific genetic band within them. This particular band might exert control over certain characteristics or traits found in these species (Fig. 2).

The genetic affinities and relatedness of the six taxa considered can be observed among some of the 28 populations (Fig. 3). Six almost distinct clusters can be observed in Fig. 3.

AMOVA results indicated that a significant proportion of the genetic variation was attributed to variation among populations, rather than within populations. These results point to restricted gene flow and possible genetic isolation within some populations, which may be caused by ecological or geographic constraints. The results of the analysis of molecular variance (AMOVA) revealed that 52% and 48% of the genetic diversity resided between and within populations, respectively. Furthermore, the genetic variation among and within populations was found to be significant at a 1% level (Table 5).

Principal Coordinate Analysis (PCOA) of R-ISSR data showed that the first three factors accounted for approximately 65.14% of the total variance. Specifically, the first, second, and third axes contributed approximately 30.36%, 17.69%, and 17.08% of the total variance, respectively. The dendrogram clustering based on Nei's unbiased measures of genetic distance produced similar results, which were supported by PCOA ordination plot (Figs. 3, 4, and Table 6).

The findings of this study showed that R-ISSR markers can be effectively utilized for genetic differentiation of *Salvia* accessions. Specifically, the primers P1 and P9 demonstrated a significant degree of polymorphism and might be useful resources for managing Salvia germplasm and conducting further breeding research.

Discussion

The experimental results of this study provide evidence for the reliability, and the usefulness of R-ISSR markers in estimating genetic diversity within and between native *Salvia* populations. The R-ISSR method effectively differentiated all species that were tested, enabling identification from the genus level to the geographical species level. Additionally, it allowed for the distinction of populations and individuals within the same species based on variations in chemical components (data not shown). The assessment of genetic diversity in the studied *Salvia* species using R-ISSR markers provides valuable insights into their evolutionary history and population dynamics in

Table 4 Overall genetic variability across 28 populations and 92 individuals in *Salvia* species

Locus	*ne**	*h**	*I**	Ht	Hs	Gst	Nm
P1-A	1.0657	0.0616	0.1411	0.0608	0.0444	0.27	1.352
P1-B	1.6955	0.4102	0.6004	0.3811	0.0887	0.7672	0.1517
P1-C	1.0128	0.0127	0.0385	0.0207	0.0148	0.2854	1.2518
P1-D	1.8427	0.4573	0.6498	0.4215	0.0914	0.783	0.1385
P1-E	1.1703	0.1455	0.2763	0.1581	0.0174	0.8897	0.062
P1-F	1.1321	0.1167	0.233	0.1612	0.0255	0.8419	0.0939
P1-G	1.9982	0.4996	0.6927	0.4894	0.1255	0.7436	0.1724
P1-H	1.141	0.1236	0.2436	0.1518	0.019	0.8749	0.0715
P1-I	1.6043	0.3767	0.5642	0.3996	0.0933	0.7664	0.1524
P1-J	1.2	0.1666	0.3065	0.2302	0.0322	0.86	0.0814
P1-K	1.137	0.1205	0.2389	0.1316	0.0488	0.6293	0.2945
P1-L	1.7274	0.4211	0.612	0.4077	0.1022	0.7493	0.1673
P1-M	1.2859	0.2224	0.3814	0.3147	0.0255	0.919	0.0441
P1-N	1.2813	0.2196	0.3778	0.2542	0.0107	0.9579	0.022
P1-O	1.7805	0.4384	0.6302	0.4103	0.1021	0.7512	0.1656
P1-p	1.3143	0.2391	0.4029	0.2437	0.0552	0.7736	0.1463
P1-q	1.9351	0.4832	0.6763	0.4765	0.223	0.5321	0.4398
P1-R	1.0128	0.0127	0.0385	0.0207	0.0148	0.2854	1.2518
P1-S	1.2242	0.1831	0.3294	0.2319	0.0834	0.6402	0.281
P1-T	1.2422	0.195	0.3454	0.2062	0.0166	0.9196	0.0437
P1-U	1.1344	0.1185	0.2358	0.1688	0.0281	0.8334	0.1
P1-v	1.1418	0.1242	0.2446	0.1583	0.0231	0.8542	0.0853
P1-w	1.4766	0.3227	0.5036	0.3436	0.1081	0.6853	0.2296
P1-X	1.1117	0.1004	0.2074	0.1153	0.0379	0.6715	0.2446
P1-Y	1.5073	0.3366	0.5194	0.3426	0.1087	0.6827	0.2324
P1-Z	1.0864	0.0796	0.1727	0.1081	0.0281	0.7399	0.1758
P1-zz	1.0279	0.0272	0.0727	0.0297	0.0174	0.4138	0.7083
P4-A	1.0945	0.0863	0.1841	0.0605	0.0447	0.2609	1.4166
P4-B	1.0231	0.0225	0.0624	0.0131	0.0118	0.1052	4.2546
P4-C	1.0894	0.082	0.1769	0.0851	0.0668	0.2148	1.8276
P4-D	1.0242	0.0236	0.0649	0.0259	0.0214	0.1727	2.3959
P4-E	1.9642	0.4909	0.684	0.4964	0.1613	0.6751	0.2406
P4-F	1.1497	0.1302	0.2536	0.1457	0.0501	0.6564	0.2618
P4-G	1.9884	0.4971	0.6902	0.4892	0.1936	0.6042	0.3276
P4-H	1.304	0.2331	0.3953	0.2973	0.0593	0.8006	0.1245
P4-I	1.6621	0.3983	0.5877	0.3997	0.1026	0.7433	0.1727
P4-J	1.2401	0.1936	0.3436	0.2546	0.0577	0.7733	0.1466
P4-K	1.3916	0.2814	0.455	0.3282	0.0327	0.9005	0.0552
P4-L	1.8932	0.4718	0.6647	0.4592	0.1773	0.614	0.3144
P4-M	1.3204	0.2427	0.4073	0.2698	0.0179	0.9338	0.0354
P4-N	1.8121	0.4481	0.6404	0.4595	0.137	0.7019	0.2124
P4-O	1.6792	0.4045	0.5943	0.347	0.1342	0.6132	0.3154
P4-p	1.3772	0.2739	0.4459	0.2352	0.1205	0.4876	0.5254
P4-q	1.037	0.0357	0.0909	0.0426	0.0338	0.2079	1.9055
P4-R	1.0258	0.0251	0.0682	0.0207	0.0148	0.2854	1.2518

Table 4 (continued)

Locus	*ne**	*h**	*I**	Ht	Hs	Gst	Nm
P4-S	1.0923	0.0845	0.181	0.0534	0.0403	0.2461	1.5318
P8-A	1.2597	0.2062	0.3603	0.2633	0.069	0.7381	0.1774
P8-B	1.0492	0.0468	0.1132	0.0518	0.0421	0.1871	2.1723
P8-C	1.2945	0.2275	0.3881	0.2649	0.1083	0.591	0.346
P8-D	1.7176	0.4178	0.6085	0.4355	0.1414	0.6752	0.2405
P8-E	1.5182	0.3413	0.5248	0.376	0.0813	0.7839	0.1379
P8-F	1.2937	0.227	0.3874	0.2788	0.0782	0.7194	0.195
P8-G	1.1524	0.1323	0.2567	0.1438	0.0464	0.6772	0.2383
P8-H	1.7872	0.4405	0.6324	0.4589	0.2214	0.5175	0.4662
P8-I	1.1842	0.1555	0.2908	0.1688	0.0281	0.8334	0.1
P8-J	1.2918	0.2259	0.386	0.2714	0.0691	0.7455	0.1707
P8-K	1.4615	0.3158	0.4955	0.3696	0.0893	0.7583	0.1594
P8-L	1.121	0.108	0.2194	0.0965	0.0174	0.8193	0.1103
P8-M	1.5429	0.3519	0.5367	0.3072	0.1436	0.5326	0.4388
P8-N	1.1661	0.1425	0.2718	0.2116	0.0214	0.8988	0.0563
P8-O	1.2833	0.2207	0.3793	0.2776	0.0717	0.7417	0.1741
P8-p	1.7281	0.4213	0.6123	0.3449	0.1676	0.5141	0.4726
P8-q	1.4487	0.3097	0.4885	0.3627	0.0717	0.8023	0.1232
P8-R	1.107	0.0967	0.2013	0.1132	0.0415	0.6338	0.2889
P8-S	1.4515	0.3111	0.49	0.3798	0.0558	0.853	0.0862
P8-T	1.3525	0.2607	0.4297	0.2083	0.1054	0.494	0.5122
P8-U	1.3514	0.26	0.4289	0.2374	0.0783	0.6703	0.2459
P8-v	1.2776	0.2173	0.3749	0.2182	0.1257	0.4239	0.6795
P8-w	1.0872	0.0802	0.1739	0.0666	0.056	0.1584	2.6575
P8-X	1.0359	0.0346	0.0887	0.0317	0.0273	0.1402	3.0652
P9-A	1.3965	0.2839	0.458	0.2543	0.0535	0.7898	0.1331
P9-B	1.1076	0.0971	0.202	0.1171	0.0429	0.6333	0.2895
P9-C	1.1402	0.123	0.2427	0.1391	0.0067	0.9515	0.0255
P9-D	1.8505	0.4596	0.6522	0.4572	0.1294	0.7169	0.1974
P9-E	1.371	0.2706	0.4419	0.3328	0.0486	0.854	0.0855
P9-F	1.1746	0.1487	0.2809	0.1457	0.0501	0.6564	0.2618
P9-G	1.5894	0.3708	0.5577	0.4128	0.1509	0.6344	0.2882
P9-H	1.1704	0.1456	0.2764	0.1719	0.0362	0.7895	0.1333
P9-I	1.7839	0.4394	0.6313	0.4504	0.1268	0.7185	0.1959
P9-J	1.4272	0.2993	0.4763	0.3466	0.0658	0.8102	0.1171
P9-K	1.8847	0.4694	0.6622	0.4589	0.1487	0.676	0.2397
P9-L	1.0965	0.088	0.1869	0.1055	0.0727	0.3109	1.1085
P9-M	1.4693	0.3194	0.4997	0.3085	0.201	0.3486	0.9341
P9-N	1.2667	0.2105	0.3661	0.2449	0.0357	0.8542	0.0854
P9-O	1.9191	0.4789	0.6719	0.4898	0.143	0.708	0.2063
P9-P	1.2175	0.1786	0.3232	0.2519	0.0497	0.8029	0.1228
P9-Q	1.5309	0.3468	0.5309	0.3161	0.0993	0.6858	0.229
P9-R	1.057	0.0539	0.1267	0.0476	0.0286	0.4004	0.7488
P9-S	1.1419	0.1243	0.2447	0.1052	0.0684	0.3503	0.9273
P9-T	1.6322	0.3873	0.5758	0.3914	0.1078	0.7246	0.1901

Table 4 (continued)

Locus	*ne**	*h**	*I**	Ht	Hs	Gst	Nm
P9-U	1.4319	0.3016	0.479	0.2536	0.0936	0.6309	0.2925
P9-v	1.3218	0.2435	0.4083	0.2602	0.1032	0.6036	0.3284
P9-w	1.1636	0.1406	0.2691	0.1327	0	1	0
Mean	1.3609	**0.2363**	**0.381**	**0.2479**	**0.074**	**0.7014**	**0.2129**
St.Dev	**0.2818**	**0.1435**	**0.187**	**0.0199**	**0.0027**		

ne* Effective number of alleles; **h* Nei's gene diversity;I* Shannon's

Information index, *Ht* Total Heterozygosity, *Hs* Expected Hetrozigosity, *Gst*: coefficient of genetic differentiation, *Nm* estimate of gene flow from Gst

Table 5 Analysis of molecular variance (AMOVA) for 28 populations of different *Salvia* species based on R-ISSR markers

Sources	df	Sum of squared deviations (SSD)	Mean square deviations (MSD)	Estimate variation	Percentage of variance
Among populations	27	777.936	28.812**	6.905	52%
Within populations	64	405.183	6.331**	6.331	48%
Total	91	1183.120		13.236	100%

**Significant at 1%, φ ST = 0.522

Iran. Based on provided results of the R-ISSR analysis for the six *Salvia* species in Iran, several hypotheses can be generated. (1) Genetic Differentiation: The observed polymorphism and genetic diversity within and among populations of *Salvia* species suggest that there is significant genetic differentiation among populations. This could be due to factors, such as geographic isolation, habitat differentiation, or reproductive barriers. (2) Population Structure: The clustering analysis, and sub-clustering patterns indicate the presence of distinct genetic clusters and subpopulations within each *Salvia* species. This suggests that certain populations have unique genetic characteristics which are genetically distinct from others. (3) Limited Gene Flow: The AMOVA results and significant genetic variation among populations suggest limited gene flow among populations, potentially affected by geographical barriers or ecological factors. Restricted gene flow may contribute to the genetic differentiation observed among *Salvia* populations. (4)Marker Utility: The primers P1 and P9, which exhibited high levels of polymorphism, can serve as valuable tools for future breeding research and germplasm management in *Salvia* species. These markers can potentially help identify specific genetic traits or characteristics of interest. 5) Genetic Affinities: The genetic affinities and relatedness observed among some populations suggest that certain *Salvia* species are more closely related to each other than to others. Evolutionary history, hybridization events could influence this pattern, or shared genetic traits within specific groups.

It's important to note that these hypotheses are based on the provided results and the information mentioned in the study. Further analyses and research would be needed to validate and explore these hypotheses in more detail.

Conservation initiatives should consider preserving the genetic integrity of these *Salvia* species, particularly by targeting populations with unique genetic characteristics or those facing significant threats. Additionally, steps should be implemented to increase gene flow across communities to decrease the hazards associated with genetic isolation and inbreeding depression. The observed heterozygosity and Nei's genetic diversity index values reflected the rich genetic reservoir harbored by these species in Iran. The reproducibility of R-ISSR markers in this study may be attributed to using longer primers and higher annealing temperatures compared to RAPD markers. R-ISSR markers exhibit simplicity and can be

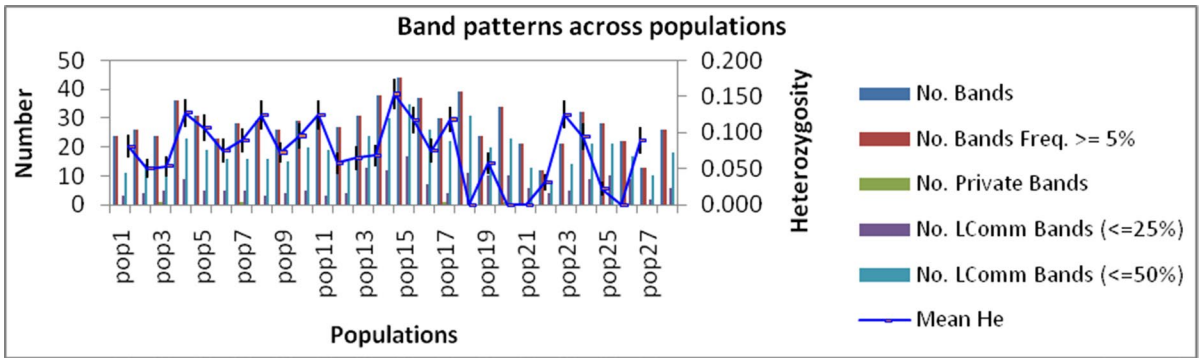

Fig. 2 Band patterns across populations based on R-ISSR data in *Salvia* species

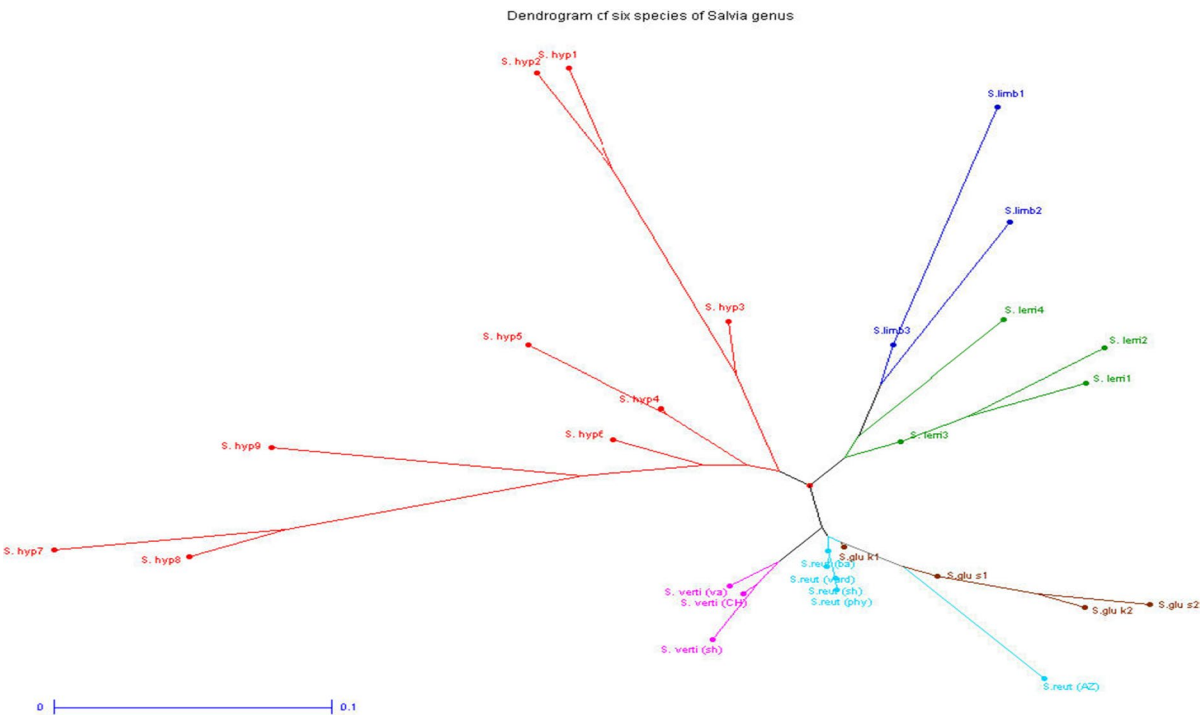

Fig. 3 Dendrogram constructed from Nei's unbiased genetic distance matrix among 28 populations (according to Table 1), clustered with the unweighted neighbor- joining (r = 0.81) based on R-ISSR data

Table 6 The cumulative percentage of population changes in three components

Component	1	2	3
% Variance	30.36	17.69	17.08
The cumulative percentage variance	30.36	48.06	65.14

used for genome mapping, gene tagging, and marker-assisted selection. Considering their high simplicity and the limited genomic knowledge for certain organisms, ISSR markers should be the primary choice for mapping or gene tagging. In cases where ISSR or RAPD alone fails to detect sufficient genetic loci, R-ISSR can be advantageous by revealing new genomic loci, requiring only the screening of suitable

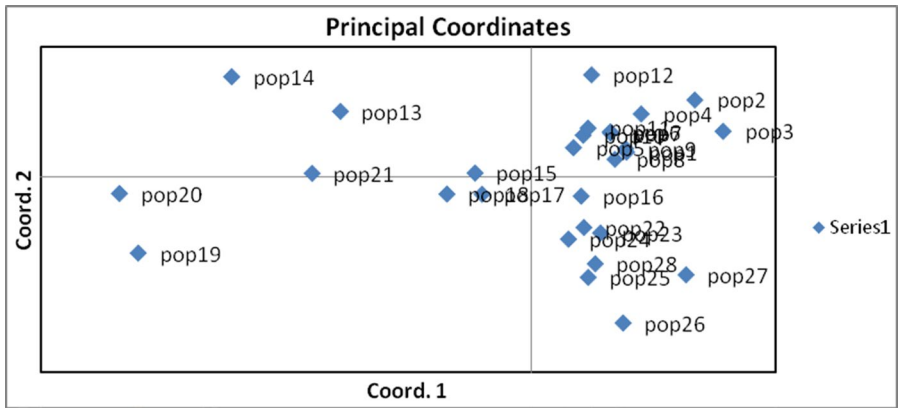

Fig. 4 Scatter plot of 28 populations (according to Table1) for the first two PCOA analyses in *Salvia* species

combinations of ISSR and RAPD primers. Comparative research using RAPD and ISSR marker systems in *Salvia* species has also been limited (Javan et al. 2012; Zhang et al. 2013a, b).

To the best of our knowledge, this study represents the first application of R-ISSR markers to characterize and evaluate genetic diversity within and among *Salvia* species. This technique offers new and interesting possibilities in the field of *Salvia* genus characterization. Additional investigation might be conducted to examine the functional consequences of the discovered genetic diversity and investigate possible associations between genetic variation and features that are ecologically or medicinally significant. Ultimately, such research will contribute to the development of sustainable management strategies and the preservation of invaluable genetic variations.

In conclusion, using R-ISSR markers proved to be a valuable tool for assessing genetic diversity and differentiation within and between populations of *Salvia* species. The markers exhibited high reproducibility and efficiency, making them suitable for genetic research and population analysis. The study successfully identified genetic clusters and relationships among the populations, providing insights into the genetic structure of *Salvia* species.

The results showed significant genetic diversity within populations, indicating the presence of a rich source of biodiversity in *Salvia* landraces. The comparison with other marker systems, such as RAPD, SRAP AFLP, SAMPL, RFLP, AFLP (Song et al. 2010; Liber et al. 2014; Rodriguez et al. 1999; Zhang et al. 2013a; Erbano et al. 2015; Bertea et al. 2006; Radosavljevic et al. 2012; Myśków et al. 2001) showed the superiority of R-ISSR markers to survey of *Salvia* species in terms of the number of markers generated, and the level of polymorphism detected (Chunjiang et al. 2005; Smolik 2012). R-ISSR markers proved to be a cost-effective alternative for molecular characterization and fingerprinting of *Salvia* species.

In general, this study highlights the importance of genetic diversity assessment in plant breeding and conservation programs. The use of R-ISSR markers offers significant contributions to the understanding of the genetic composition and interconnections across populations of Salvia species. Further research can build upon these findings to expand the study of genetic diversity in additional *Salvia* species and populations, contributing to broader understanding and conservation of this diverse genus.

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Declarations

Conflict of interest The author declares that he has no conflicts of interest with respect to the research, authorship and publication of this manuscript.

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